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Distribution and Functions of Phosphotransferase System Genes in the Genome of the Lactic Acid Bacterium *Oenococcus oeni*

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***Oenococcus oeni*, the lactic acid bacterium primarily responsible for malolactic fermentation in wine, is able to grow on a large variety of carbohydrates, but the pathways by which substrates are transported and phosphorylated in this species have been poorly studied. We show that the genes encoding the general phosphotransferase proteins, enzyme I (EI) and histidine protein (HPr), as well as 21 permease genes (3 isolated ones and 18 clustered into 6 distinct loci), are highly conserved among the strains studied and may form part of the *O. oeni* core genome. Additional permease genes differentiate the strains and may have been acquired or lost by horizontal gene transfer events. The core *pts* genes are expressed, and permease gene expression is modulated by the nature of the bacterial growth substrate. Decryptified *O. oeni* cells are able to phosphorylate glucose, cellobiose, trehalose, and mannose at the expense of phosphoenolpyruvate. These substrates are present at low concentrations in wine at the end of alcoholic fermentation. The phosphotransferase system (PTS) may contribute to the perfect adaptation of *O. oeni* to its singular ecological niche.**

Oenococcus oeni (formerly *Leuconostoc oenos*) is an alcohol-tolerant, acidophilic lactic acid bacterium primarily responsible for malolactic fermentation in wine (1). Moreover, selected *O. oeni* strains have been shown to display immunomodulatory potential, which opened new opportunities for the use of these wine bacteria as live probiotics (2).

O. oeni is able to grow on a large variety of carbohydrates (3), but the metabolic pathways by which the carbohydrates are converted to energy have been investigated only for glucose and fructose (4, 5). These hexoses are converted to energy via the phosphoketolase pathway, leading to the excretion of lactate, ethanol, or acetate and CO_2 as the main products (4–7). Even fewer studies have focused on the transport capabilities of this bacterial species (8). The percentage of recognized genes that are predicted to encode transport proteins in *O. oeni* PSU-1 genome is the highest among the low-GC lactic acid bacteria analyzed by Lorca et al. (8). Among the genes encoding proteins dedicated to carbohydrate transport, 32% are associated with the phosphoenolpyruvate (PEP) phosphotransferase system (PTS). PTS proteins are group translocators that catalyze the uptake of hexoses or hexose derivatives (sugar alcohols, sugar amines. . .) and also their concomitant phosphorylation at the expense of PEP (9). PTS systems consist of an integral membrane protein (enzyme IIC [EIIC]) that translocates the substrate across the membrane and several cytoplasmic proteins that transfer the phosphoryl group (P) and the energy from PEP to the substrate (10):

$$\text{P-enolpyruvate} + \text{EI} \rightleftarrows \text{P-EI} + \text{pyruvate} \quad (1)$$

$$\text{P-EI} + \text{HPr} \rightleftarrows \text{P-HPr} + \text{EI} \quad (2)$$

$$\text{P-HPr} + \text{EIIA} \leftrightarrows \text{P-EIIA} + \text{HPr} \quad (3)$$

$$\text{P-EIIA} + \text{EIIB} \rightleftarrows \text{P-EIIB} + \text{EIIA} \quad (4)$$

$$\text{P-EIIB} + \text{substrate (out)} \xrightarrow{\text{EIIC}} \text{Substrate-P (in)} + \text{EIIB} \quad (5)$$

Alternately, EI may be phosphorylated in the presence of ATP and acetate kinase (11). EI and histidine protein (HPr) are used in the phosphorylation cascade for all the PTS substrates in a cell. In contrast, EII(s) are substrate-specific permeases that consist of three or four domains IIA, IIB, and IIC (present in all families) and IID (present only in the mannose family). They can be encoded by a single gene (IIABC) or by separated ones (IIA, IIB, IIC, IIBC, etc. [10]).

PTS confers an advantage for the rapid uptake of sugar after a starvation period (10, 12, 13). In addition, specific regulatory roles have been ascribed to protein components of the PTS, for the control of carbohydrate metabolism but also for the regulation of biofilm formation, stress response, gut colonization, chemotaxis, or virulence (14–24). This emphasized the importance of PTS in bacterial metabolism regulation, beyond its primary function in transport and phosphorylation.

Glucose transport and phosphorylation through PTS was recently measured in *O. oeni* B1 (25). The PTS^{Glu} (PTS transporting glucose) phosphorylation cascade in this heterofermentative microorganism is thus known. The goals of our work were to examine (i) whether the PTS genes other than PTS^{Glu} identified in *O. oeni* PSU-1 and ATCC BAA-1163 genomes were widespread in the *O. oeni* species, (ii) how the *O. oeni* strain studied has acquired or lost these genes, (iii) whether these genes were expressed and when, and (iv) whether permeabilized *O. oeni* cells were able to phosphorylate carbohydrates other than glucose at the expense of PEP.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were *Oenococcus oeni* PSU-1 and *Lactococcus lactis* IL1403. *O. oeni* was grown at 25°C without agitation in half diluted MRS medium containing the following (concentrations shown in gram · liter^{-1}): yeast extract, 2; beef extract, 4; Bacto peptone, 5; sodium acetate, 2.5; trisodium citrate, 1; K_2HPO_4, 1; $MgSO_4 \cdot 7\ H_2O$, 0.1; $MnSO_4 \cdot H_2O$, 0.05; Tween 80, 0.5 ml. The carbon source used was either glucose (10 g · liter^{-1}), trehalose (10 g · liter^{-1}), cellobiose (10 g · liter^{-1}), arabinose (10 g · liter^{-1}), or mannose (10 g · liter^{-1}). Before sterilization (20 min, 121°C, 1 bar), the initial pH was adjusted with HCl to study PTS activities after (i) growth under acidic conditions (pH 4.7), (ii) growth at optimal pH (pH 5.5), and (iii) growth under nonacidic conditions (pH 7.0). When stated in the text, ethanol (10% [vol/vol]) was added to the medium. *L. lactis* was grown at 30°C, without agitation, in M17 broth (26) containing 0.2% glucose.

Genome analysis. Fourteen *O. oeni* genome sequences are currently available: the completed genome sequence CP000411 of strain PSU-1, which will be used as the model strain for this study and 13 partially annotated drafts corresponding to strains ATCC BAA-1163 and AWRIB-129, -202, -304, -318, -418, -419, -422, -429, -548, -553, -568, and -576 (GenBank accession numbers AAUV00000000, AJTP00000000, AJTO00000000, AJIJ00000000, ALAD00000000, ALAE00000000, ALAF00000000, ALAG00000000, ACSE00000000, ALAH00000000, ALAI00000000, ALAJ00000000, and ALAK00000000). The genome draft of *Oenococcus kitaharae* DSM 17330 (GenBank accession number AFVZ00000000) was also examined.

The phosphotransferase system (PTS) genes were identified by protein sequence analysis and comparison with databases using NCBI BLAST2 software (27). Cross searches between genomes were also carried out using BLASTN. When orthologs were present in *O. oeni* strains, the identity was higher than 90%. The *pts* genes found in *O. kitaharae* displayed at least 80% identity with their *O. oeni* orthologs. The permease substrate specificity was attributed to homology with biochemically characterized PTS transporters in other species, and the transporters were classified according to the Transport Classification Database (TCDB) (http://www.tcdb.org/).

DNA and RNA extraction. Total genomic DNA of lactic acid bacteria was purified using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) as previously described (28). For total RNA extraction, cells were harvested by centrifugation (6,000 × *g*, 15 min), suspended in Tri reagent (Sigma), and immediately disrupted with glass beads (0.1 mm) in a FastPrep FP120 instrument at 4°C for six 45-s pulses at 6,500 × *g*. Cell debris was eliminated by centrifugation, and RNA was purified from the supernatant by chloroform extraction. RNA was then precipitated using isopropanol, washed with 80% ethanol, and finally resuspended in diethylpyrocarbonate (DEPC)-treated water. RNA concentration was calculated from the absorbance measured at 260 nm (SmartSpec Plus spectrophotometer; Bio-Rad). Samples were treated with DNase as indicated by the manufacturer (DNaA-free; Ambion). Absence of chromosomal DNA was controlled by PCR using OO1/OO2 primers or any other primer set in Table S1 in the supplemental material. For cells grown in grape juice, in order to avoid interference of tannins or polyphenol on cDNA synthesis, Tri reagent was replaced by buffer containing the following: 300 mM Tris-HCl (pH 8), 25 mM EDTA (pH 8), 2 M NaCl, 2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinylpolypyrrolidone (PVPP), 0.05% spermidine, and 2% β-mercaptoethanol. The quality of RNA samples was checked on a high-resolution 1% formaldehyde-agarose gel (ethidium bromide stained). cDNA was then synthesized using the iScript cDNA synthesis kit (Bio-Rad), as recommended by the manufacturer.

Reverse transcription-PCR (RT-PCR) analysis. The 50-μl reaction mix contained 25 μl of the 2× SYBR green PCR supermix (Bio-Rad, USA), 5 pmol of the relevant primers (see Table S1 in the supplemental material), and 1 μl of cDNA in the appropriate dilution. The reaction mixture was preheated for 5 min at 95°C, and 35 cycles (denaturing step of 30 s at 95°C, annealing step of 30 s at the hybridization temperature indicated in Table S1, and extension step of 30 s at 72°C) were carried out. After the last cycle, a melting curve analysis was performed using the iCycler iQ (Bio-Rad) to check PCR specificity. The results were analyzed using the comparative critical threshold method with the *O. oeni ldhD* and *gyrA* genes as an internal calibrated target, as proposed by Desroches et al. (29) for this microorganism. Template-free reaction mixtures were included as controls. Measurements were done in triplicate, as recommended by Udvardi et al. (30).

Cell concentration and substrate and product analyses. Cell growth was estimated through the measurement of the cell suspension absorbance (optical density at 600 nm [OD_{600}]). Carbohydrate, lactate, acetate, and ethanol concentrations in the sample supernatants (10,000 × *g*, 4°C, 5 min) were measured by anion-exchange chromatography (Aminex HPX87H column; Bio-Rad) using a Waters (Milford, MA, USA) system consisting of a pump (Waters 600), an injector (Waters 717), and a refractometer (Waters 2414). The eluant (5 mM H_2SO_4) had a constant flow rate of 0.5 ml · min^{-1} at room temperature (31).

Activity measurements. Enzyme activities were measured with freshly prepared permeabilized cells obtained by a modified version of the method of Kornberg and Reeves (32). The cells were harvested at the end of the exponential growth phase by centrifugation (10,000 × *g*, 5 min, 4°C) and washed twice with cold NaCl (9 g · liter^{-1} at 4°C). To obtain permeabilized cells, the pellet was resuspended in PTS buffer containing K_2HPO_4 (50 mM; pH 6.5), PEG 8000 (67 g · liter^{-1}), and $MgCl_2$ (10 mM) in a volume sufficient to ensure an OD_{600} between 5 and 15. The cells were permeabilized by vortexing (3 min) after the addition of 0.1 volume of toluene-ethanol (1:9). Then, the permeabilized cells were washed twice with PTS buffer in order to lower the background activities. The OD_{600} of the permeabilized cell suspension was measured to standardize the assays, and correlations were used to convert the absorbance to a cell (dry weight) concentration (1 unit OD_{600} corresponded to 0.30 g [cell dry weight] · liter^{-1}). We verified by microscopic observation and using fluorescent probes (Syto9 [6 μM] and propidium iodide [60 μM]; Invitrogen, France) that this resulted in cell permeabilization and not in cell disruption. Lactate dehydrogenase activity measurements confirmed that this treatment made accessible the intracellular activities.

The permeabilized cells were maintained on ice and were used within 3 h following their preparation. The various enzyme activities were evaluated spectrophotometrically (4) in 1-cm-light-path cuvettes containing 1 ml of reaction medium by measuring the appearance/disappearance of NAD(P)H at 340 nm ($\varepsilon = 6.22 \times 10^3\ M^{-1} \cdot cm^{-1}$).

Glucokinase was measured through the formation of NADPH (31) with the following reaction mixture: 50 mM K_2HPO_4 (pH 6.5), 5 mM $MgCl_2$, 0.5 mM NADP, 1 mM ATP, glucose-6-phosphate [glucose-6-P] dehydrogenase (2 U · ml^{-1}), and cell extract or permeabilized cells. The addition of glucose (10 mM) started the reaction. The lactate dehydrogenase (EC 1.1.1.27) activity was evaluated by measuring the disappearance of NADH under the following conditions: 50 mM Tris-HCl buffer (pH 7.0), 5 mM $MgCl_2$, 0.2 mM NADH, and cell extract or permeabilized cells. The addition of pyruvate (50 mM) started the reaction.

Phosphotransferase system assays were performed by a modified version of the method of Kornberg and Reeves (32). Reaction mixtures consisted of 50 mM K_2HPO_4 buffer (pH 7.0), 1 mM PEP, 5 mM $MgCl_2$, 0.2 mM NADH, 5 U lactate dehydrogenase, and permeabilized cells. After 10 min of preincubation, the substrate (0.2 to 10 mM) was added to start the reaction. Control assays lacking PEP and/or substrate were included to account for background NADH transformation. We also checked that the activity measured was proportional to the quantity of cells introduced in the cuvette. One unit of activity was the amount of enzyme required to convert 1 nmol of substrate per min.

Statistical analysis. The statistical significance of differences between means was calculated by analysis of variance (ANOVA) followed by Tukey posthoc comparisons.

TABLE 1 Inventory of the PTS genes conserved in the 14 *O. oeni* publicly available genomes[a]

Operon identifier[b]	Protein predicted function[c]	*O. oeni* PSU-1		*O. oeni* ATCC BAA-1163		No. of strains displaying the gene ($n = 14$)	Conclusion	TCDB classification[f]
		Gene (OEOE_)[d]	Protein size (aa)	Ortholog (OENOO_)[d]	Protein size (aa)[e]			
	HPr	0643	87	38010		14	Core	
	EI	0647	575	38006		14	Core	
celA	$IIC^{cellobiose}$	0221	450	52046		14	Core	4.A.3
	$IIA^{cellobiose}$	0222	107	52045		14		
	$IIB^{cellobiose}$	0223	102	52044		14		
	Pβ-glucosidase	0224	480	52043		14		
**	$IIA^{mannose/fructose}$	0233	137	52032		14	Core	4.A.2
galA	$IIA^{galacticol}$	0234	153	52031		14	Core	4.A.5
	$IIC^{galacticol}$	0235	445	52030*		14		
	$IIB^{galacticol}$	0236	94	52029		14		
**	$IIC^{cellobiose}$	0282	401	66127*		14	Core	4.A.3
celB	$IIA^{glucose}$	0296	169	66114		14	Core	4.A.1
	$IIBC^{\beta\text{-glucoside}}$	0297	236	66113	482[g]	14		4.A.3
	$IIA^{\beta\text{-glucoside}}$	0298*				14		4.A.3
celC	$IIB^{cellobiose}$	0338	102			8		4.A.3
	$IIA^{cellobiose}$	0339	111			8		
	6P-β-glucosidase	0340	481			8		
	6P-β-glucosidase	0341	485			8		
	$IIC^{cellobiose}$	0342*				8		
	$IIC^{cellobiose}$	0343	433			8		
**	$IIBC^{fructose}$	0363	321	66080	344	14	Core	4.A.2
manA	$IIB^{mannose}$	0379	163	66064*		14	Core	4.A.6
	$IIC^{mannose}$	0380	311	66063		14		
	$IID^{mannose}$	0381	272	66062		14		
	$IIA^{mannose}$	0382	139	66061		14		
manB	$IIAB^{mannose}$	0464	331	40010		14	Core	4.A.6
	$IIC^{mannose}$	0465	271	40009		14		
	$IID^{mannose}$	0466	304	40008		14		
**	$IIC^{cellobiose}$			40004	195	1		
manC	$IIA^{mannose}$	1204	100	27004*		6		4.A.2
	$IIB^{fructose/mannose}$			27003	165	5		
	$IIC^{mannose}$			27002	286	3		
	$IID^{mannose}$			27001*		3		
celD	$IIA^{cellobiose}$	1207	107	27006		3		4.A.3
	$IIB^{cellobiose}$	1208	102	27007		3		
	$IIC^{cellobiose}$	1209*		27008	442	14		
	6P-β-glucosidase	1210	440	27009*		14		
treA	P-trehalase	1340	553	49033*		14	Core	4.A.1
	$IIBC^{trehalose}$	1341	503	49034		14		
	$IIA^{glucose/trehalose}$	1342	162	49035		14		
ascA	$IIC^{ascorbate}$	1481*		63003	456	12		4.A.7
	$IIB^{ascorbate}$	1482	99	63004		12		
	$IIA^{fructose}$	1483	160	63005		12		

(Continued on following page)

TABLE 1 (Continued)

Operon identifier[b]	Protein predicted function[c]	*O. oeni* PSU-1 Gene (OEOE_)[d]	*O. oeni* PSU-1 Protein size (aa)	*O. oeni* ATCC BAA-1163 Ortholog (OENOO_)[d]	*O. oeni* ATCC BAA-1163 Protein size (aa)[e]	No. of strains displaying the gene ($n = 14$)	Conclusion	TCDB classification[f]
fruA	$IIBC^{fructose}$			63017	483	6		4.A.2
	$IIA^{fructose}$			63018	180	6		
sucA	$IIA^{glucose/sucrose}$			63038		1		4.A.1
	Suc-6P-hydrolase			63039		1		
	$IIC^{sucrose}$			63040		1		

[a] The PTS genes were searched for in the genome sequences of *O. oeni* PSU-1 and ATCC BAA-1163, and then, orthologs were searched for in the 12 AWRI strains.
[b] **, isolated gene.
[c] The predicted functions of the proteins are shown. $IIC^{cellobiose}$, PTS transporter IIC specific for cellobiose.
[d] Pseudogenes are indicated by an asterisk after the gene number. Genes whose expression was checked by quantitative RT-PCR (qRT-PCR) are underlined.
[e] If the protein size was significantly different from that of strain PSU-1.
[f] Family in the membrane protein Transporter Classification Database (TCDB) (http://www.tcdb.org/): 4.A.1, the $PTS^{glucose\text{-}glucoside}$ (Glu) family; 4.A.2, the $PTS^{fructose\text{-}mannitol}$ (Fru) family; 4.A.3, the $PTS^{lactose}$ (Lac) family; 4.A.4, the $PTS^{glucitol}$ (Gut) family; 4.A.5, the $PTS^{galacticol}$ (Gal) family; 4.A.6, the $PTS^{mannose\text{-}fructose\text{-}sorbose}$ (Man, requiring IID) family; 4.A.7, the PTS^{L}-ascorbate (L-Asc) family.
[g] Gene OENOO_63113 matches with both genes OEOE_0297 and OEOE_0298 and encodes a IIABC β-glucoside permease of 482 aa.

RESULTS AND DISCUSSION

***pts* gene inventory in publicly available *O. oeni* genomes.** The *ptsI* and *ptsH* genes encoding the general PTS proteins EI and HPr and a total of 40 PTS permease genes were found in *O. oeni* PSU-1 and ATCC BAA-1163 genome sequences. These 42 genes were then searched in the 12 *O. oeni* AWRIB strain genome sequences (Table 1). *pts* genes specific to AWRIB strains are not listed in Table 1.

Only four out of the 40 PTS permease genes were isolated genes, while the remaining ones were grouped into gene clusters whose names were chosen depending on the most probable substrate specificity of the PTS permeases.

The *ptsI* and *ptsH* genes were highly conserved in the *O. oeni* species (over 99% identity between the 14 strains). Moreover, the peptides bearing the key amino acids involved in protein phosphorylation (i.e., His 15 or Ser 46 on HPr and His 189 on EI) were also highly conserved, compared with those of enzymes of other species (10, 33). However, *ptsI* and *ptsH* did not form part of an operon in *O. oeni*, in contrast to what was described for other lactic acid bacteria, either homofermentative or heterofermentative (Fig. 1A and B). The same organization was found in *Oenococcus kitaharae*, the only other species in the genus *Oenococcus* and could be specific to this genus among the *Lactobacillalles*.

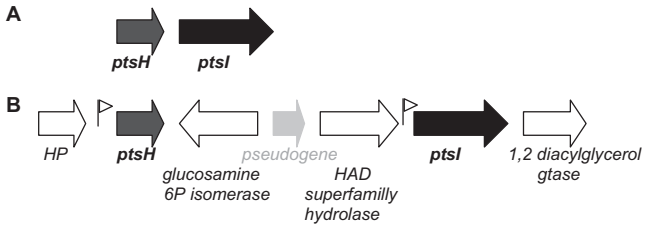

FIG 1 Organization of the general *pts* genes in *Lactobacillus* (A) and *Oenococcus* (B) species. (A) Organization of the general *pts* genes in *Lactobacillus johnsonii* NCC 533, *Lactobacillus hilgardii* ATCC 8290, *Lactobacillus sakei* 23K, *Lactobacillus plantarum* WCF51, *Lactococcus lactis* IL1403, *Leuconostoc mesenteroides* ATCC 8293, and *Lactobacillus casei* ATCC 334. (B) Organization of the general *pts* genes in *O. oeni* strains PSU-1, ATCC BAA-1163, and AWRI B-429 and *O. kitaharae* DSM17330. Putative promoters are represented as small white flags. HP, hypothetical protein; HAD, haloacid dehalogenase.

The complete set of permeases (IIA plus IIB plus IIC plus when appropriate, IID for PTS mannose [PTS^{Man}]) was encoded by most of the loci mentioned in Table 1, whatever the genotype of the strain: *celA*, *celC*, *galA*, *manA*, *manB*, *treA*, and *fruA* strains. The other loci contained either an incomplete set of permease genes (*sucA*) or a complete set of genes with some pseudogenes (*celB*, *manC*, and *celD*) between the permease genes. The nucleotide sequence and the synteny were highly conserved for the various *pts* loci (see Fig. S1 in the supplemental material). Furthermore, in addition to the PTS permease set, *O. oeni pts* loci often displayed a transcriptional regulator and, when appropriate, the glycosidase required for channeling the phosphorylated disaccharide substrate to the central metabolic pathways:

$$\text{Trehalose} \xrightarrow{PTS^{Tre}} \text{trehalose-P} \xrightarrow{\text{phosphotrehalase}} \text{glucose-P} + \text{glucose} \quad (6)$$

$$\text{Cellobiose} \xrightarrow{PTS^{Cel}} \text{cellobiose-P} \xrightarrow{\text{P-β-glucosidase}} \text{glucose-P} + \text{glucose} \quad (7)$$

The *pts* gene sequence analysis suggests that hexoses (fructose and mannose), hexose derivatives (galacticol and mannitol), vitamins (ascorbate), and oligosaccharides made of hexoses (cellobiose, trehalose, and sucrose) are PTS substrates for *O. oeni*. The list of PTS substrates may be longer, since most PTS permeases were shown to be able to phosphorylate several substrates (10). Moreover, substrate specificity is sometimes difficult to predict from sequence comparisons (34, 35) However, the presence of the phospho-sugar-hydrolase genes brought further insights on the substrate specificity of the *celA*, *celC*, and *treA* loci.

Acquisition of the PTS permease genes. The *ptsI* and *ptsH* genes, 3 isolated permease genes, and the 18 permease genes clustered into 6 *pts* loci (*celA*, *galA*, *celB*, *manA*, *manB*, and *treA*) were present in the 14 genomes examined and may form part of the core genome of *O. oeni*. Additional PTS loci (*celC*, *ascA*, *manC*, *fruA*, and *sucA*) were also specifically found or present in some of the strains studied, while either the entire *celD* locus or a truncated form was present depending on the strain (Table 1).

The sequences flanking the various *pts* genes and loci were analyzed. The possible position of each *pts* locus in the pangenome

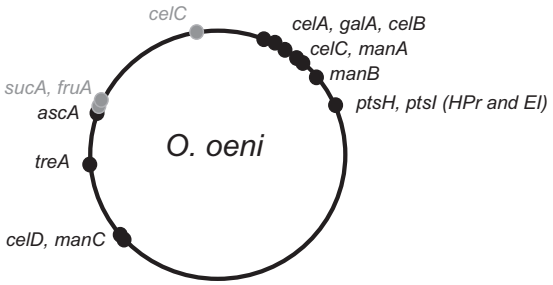

FIG 2 Schematic representation of the various *pts* loci on the chromosome of *O. oeni*. The genome of *O. oeni* PSU-1 is represented with its 10 *pts* loci (black). The positions of the adjacent regions of the 2 additional loci found in *O. oeni* ATCC BAA-1163 and the insertion site of the *celC* locus in strains other than PSU-1 are presented in gray.

of *O. oeni* is represented in Fig. 2. All the core *pts* genes and most of the additional *pts* genes were inserted in the same position in the chromosomes of all the strains bearing them, with the exception of *celC*, which displayed two distinct insertion sites, depending on the strain considered.

The *pts* loci *celA*, *treA*, *manA*, and *manB* were also found in *O. kitaharae* (more than 80% identity, with conserved synteny). The isolated permeases $IIA^{Man/Fru}$ (OEOE_0233) and IIA^{Fru} (OEOE_1204) were also present in the *O. kitaharae* genome sequence. As already mentioned for *ptsH* and *ptsI*, the *pts* loci *celA*, *treA*, *manA*, and *manB* and the two isolated permeases mentioned above may have been inherited from the *Oenococcus* genus common ancestor. On the other hand, the *O. oeni* core *pts* loci *celB* and *galA* were absent in the *O. kitaharae* genome. As suggested earlier (36), these specific *pts* genes may have been acquired by horizontal gene transfer and have stabilized very early in the *O. oeni* species evolution. We did not find any trace of the mobile element that could be at the origin of the *celB* or the *galA* locus integration in the *O. oeni* chromosome.

The *pts* permease genes that were specifically present in certain strains may have been acquired later. The *sucA*, *fruA* and *ascA* loci were located in the hypervariable region also bearing exopolysaccharide genes previously mentioned by Borneman et al. (37) and Dimopoulou et al. (38). This region also displayed other *pts* genes specific to AWRIB strains (37). Many recombination events involving insertion sequences may be at the origin of this mosaic region.

The *celC* locus was found in strains AWRIB-202, -304, -318, -419, -429, -422, -548, -568, and -576 in the form of a 10.2-kb insert (see Fig. S2 in the supplemental material). This chromosome region contained two tRNAs, which suggests that a bacteriophage could be at the origin of the 10.2-kb insert integration in the chromosome, though no phage-specific genes were found in this chromosome area (39). The *celC* locus was also found in *O. oeni* PSU-1 in a 24.6-kb insert containing several transposases, which may be at the origin of the insert construction and integration. Interestingly, the 24.6-kb insert integration site encroached upon a core *pts* gene encoding a $IIBC^{Fru}$ permease subunit (gene OEOE_0363). The 5′ end of this gene is truncated in *O. oeni* PSU-1, compared with other strains, such as *O. oeni* ATCC BAA-1163 (Table 1).

Figure 3 depicts the organization of the region flanking the *celD* and *manC* loci in strains that have these genes or do not have these genes. The *celD* and *manC* loci, separated by *deoC* also appeared to be targets for or collateral victims of indel events in the *O. oeni* chromosome. The *manC* locus was complete (with all permease genes present) in strains AWRIB-419 and ATCC BAA-1163. The *manC* locus was present in a truncated form in strains AWRIB-129, AWRIB-422, and PSU-1 and was absent in strains AWRIB-429 and AWRIB-553. The complete *celD* locus was present only in

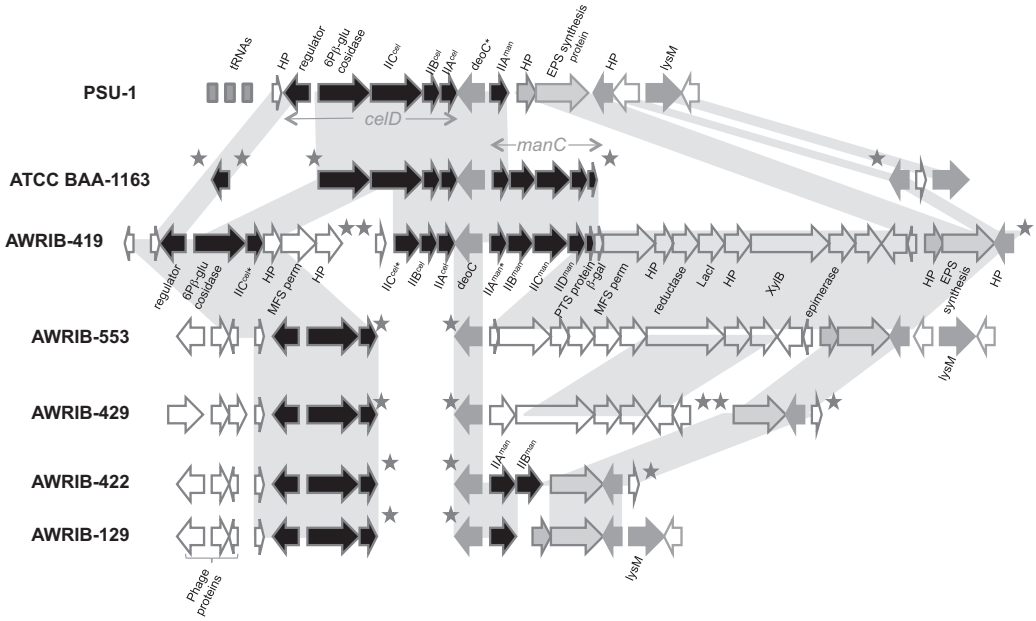

FIG 3 Schematic representation of the chromosome region containing the accessory *pts* loci *manC* and *celD* in some of the strains studied. This region is a genome assembly hot spot (the ends of contigs are indicated by stars), which suggests that indel events are frequent in this chromosome area. The central gene *deoC* and the gene encoding the LysM protein are present in all the strains studied. Pseudogenes are indicated by asterisks. EPS, extracellular polymeric substance; MFS perm, major facilitator superfamily permease.

O. oeni strain PSU-1, next to a tRNA locus, suggesting that phage integration may occur near this *pts* locus. Indeed, phage proteins are encountered next to *celD* in strains AWRIB-419, -553, -429, -422, and -129 in which the locus is truncated. In strain AWRIB-419, an insertion event in the permease IIC gene was clearly at the origin of the *celD* locus truncation. Similar events may also have occurred in strains AWRIB-553, -429, -422, and -129. Actually, the genes encoding the permease subunit IIC and the 6-phosphate-β-glucosidase (6-P-β-glucosidase) were present in the 14 strains studied, while the genes encoding IIA and IIB were missing in most of the other strains (Table 1).

Expression of the *pts* gene. The expression of *ptsI* and *ptsH* genes and of several genes in different *pts* loci (*celA*, *galA ceiC*, *manA*, *manB*, and *treA*) was studied by real-time quantitative RT-PCR. *O. oeni* PSU-1 was chosen as the model strain. Several culture parameters were varied: the carbon substrate (arabinose, glucose, cellobiose, trehalose, mannose, or grape juice), the initial pH of the culture medium (4.7, 5.5, or 7.0), and the absence or presence of ethanol (10%). The cultures were grown in liquid or solid medium. During planktonic growth, the different genes studied appeared slightly overexpressed at the entry into stationary growth phase compared to their level of expression in the exponential growth phase or late stationary phase, whatever the growth substrate, ethanol concentration, or broth pH (see the example in Fig. 4).

The *ptsH* gene (HPr) was expressed at levels comparable to those of housekeeping genes, while *ptsI* (EI) was significantly less expressed. The *ptsH/ptsI* expression ratio varied from 3 to 20, depending on the growth conditions. This ratio was similar to that reported for other Gram-positive bacteria with low GC content (between 5 to 100 according to reference 40), although the organization of these genes is very different in *O. oeni*.

The influence of the growth substrate on *pts* gene expression was studied, taking as the reference culture, a culture in liquid medium in the presence of arabinose, a substrate not transported by the PTS system (Fig. 5). The *ptsH* and *ptsI* genes were slightly overexpressed in the presence of substrates other than arabinose (potent PTS substrates). The *celA* locus was highly overexpressed in the presence of cellobiose and overexpressed to a lesser extent in the presence of trehalose. The *galA* locus was slightly overexpressed in the presence of grape juice. The *manA* and *manB* loci were overexpressed in the presence of glucose and mannose, but grape juice, which is rich in glucose and fructose, stimulated the expression of *manA* and had no effect on *manB*. This may not be linked to the presence of fructose but rather to other elements in grape juice, since fructose was shown to stimulate the expression of *manB* and not *manA* by Kim et al. (25). The *treA* locus was strongly overexpressed in the presence of trehalose. In contrast, no substrate specificity could be proposed for the enzyme encoded by the *celC* locus. However, *celC* was underexpressed in the presence of grape juice. Many elements specifically present in grape juice could be responsible for the effect observed (fructose, tartaric acid, and tannins or polyphenols in grapes).

Glucose present in MRS-glucose (MRS medium with glucose as the carbon source) and grape juice-based medium did not seem to have a negative effect on the expression of genes in the *celA*, *galA*, *manA*, *manB*, and *treA* loci, compared to arabinose. We did not observe catabolic repression for the *pts* genes.

Expression of the *pts* genes was not different when the cells were grown on solid medium instead of liquid medium (not shown). In the presence of ethanol, the level of expression of the *pts* genes studied increased slightly (1.5-fold [data not shown]). The initial pH of the culture medium also modulated the expression of certain *pts* genes (Fig. 6). The *manB* and *treA* loci were more efficiently expressed when the medium pH was above 5.0 than at acidic pH. In contrast, the *manA* locus is better expressed at acidic than at neutral pH. These results are consistent with those obtained by Kim et al. (25) for the two *man* loci (*manA* and *manB*). For the other *pts* genes studied, no effect of pH was observed on the expression of the genes.

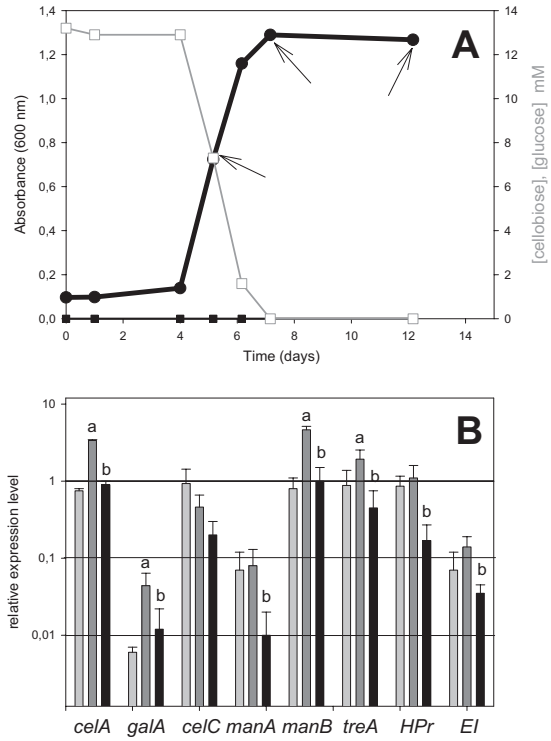

FIG 4 (A) *O. oeni* PSU-1 growth and cellobiose consumption in the absence of alcohol and with an initial medium pH of 4.7. Absorbance is shown by the thick black line with black circles. The concentrations (in millimolar) of cellobiose (large white squares) and of glucose (small black squares) are shown. The arrows indicate the samples for real-time PCR assay. (B) Relative levels of expression of various core PTS genes in cells in different growth phases. The growth phase of the cells is shown as follows: exponential growth phase (light gray bars), early stationary phase (dark gray bars), and late stationary phase (black bars). The gene studied for each locus is indicated in Table 1 and Table S1 in the supplemental material. Values that were significantly different ($P < 0.05$ by the Student *t* test) from the level of expression for the same gene in cells during exponential phase are indicated by the letter a above the bar, while values that were significantly different ($P < 0.05$ by the Student *t* test) from the level of expression for the same gene in cells in early stationary growth phase are indicated by the letter b above the bar.

PEP-dependent phosphorylation assays. We then analyzed the PTS activity of *O. oeni* PSU-1 cells (Table 2). *O. oeni* PSU-1 cells, grown in the presence of trehalose, exhibit significant PTS^{Tre} (Tre stands for trehalose), PTS^{Cel} (Cel stands for cellobiose), and PTS^{Glu} activities but show no significant PTS activity in the presence of mannose or 2-deoxyglucose. *O. oeni* PSU-1 cells grown on cellobiose had significant PTS^{Cel} and PTS^{Glu} activities, and after growth on glucose or mannose, *O. oeni* PSU-1 cells displayed significant PTS^{Tre}, PTS^{Glu}, PTS^{Cel}, and PTS^{Man} activities. No PTS activity was detected after culture in the presence of arabinose.

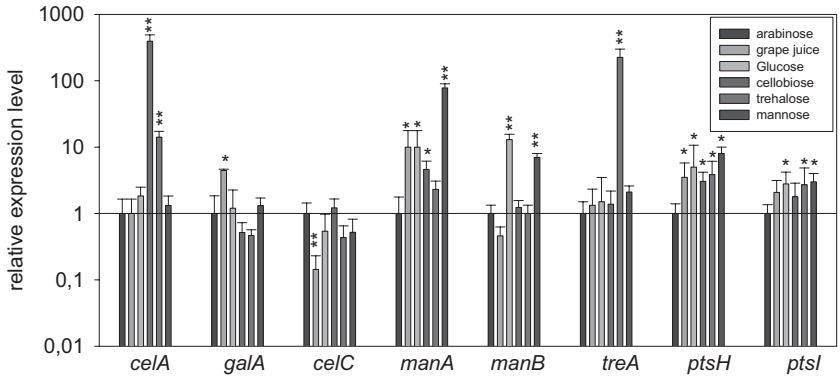

FIG 5 Influence of the growth substrate on relative expression of *pts* genes. *O. oeni* PSU-1 was grown without alcohol, and the initial pH was set at 4.7. For each *pts* gene shown in the figure, the *O. oeni* PSU-1 strain was grown with six different substrates; from left to right, the strain was grown with arabinose, grape juice, glucose, cellobiose, trehalose, and mannose. The PSU-1 strain grown on medium containing arabinose (arabinose is not a PTS substrate) was used as the control, and the expression level was set at 1. The gene studied for each locus is indicated in Table 1 and Table S1 in the supplemental material. Values that were significantly different from the level of expression of the same gene in cells after growth on arabinose are indicated by asterisks as follows: *, $P < 0.05$ by the Student *t* test; **, $P < 0.005$ by the Student *t* test.

PTS^{Fru} (Fru stands for fructose) activities could not be measured by the method employed due to high background signal due to NADH consumption by mannitol dehydrogenase in the presence of fructose. The nature of the substrates recognized as PTS substrates and significantly phosphorylated in the presence of PEP was strongly dependent on the nature of the growth substrate. Cellobiose-grown cells presented the highest PTS^{Cel} activity, mannose-grown cells presented the highest PTS^{Glu} and PTS^{Man} activities, and trehalose-grown cells presented the highest PTS^{Tre} activity.

Whatever the growth substrate, a significant glucokinase activity was measured with decryptified *O. oeni* cells (Table 2). The ATP-dependent phosphorylation of glucose was of the same order of magnitude as the PEP-dependent phosphorylation activity (PTS^{Glu}). The same results were observed with *L. lactis* IL1403 grown on glucose. PTS^{Glu} and glucokinase activities measured in *O. oeni* were noticeably lower than those measured with *L. lactis*. However, it seems important to consider the respective rate of growth of the microorganisms studied: *O. oeni* has a maximum growth rate of 0.06 h^{-1}, which is about 10 times lower than that of *L. lactis*. Therefore, it displays a much lower overall metabolic activity.

Conclusion. Among lactic acid bacteria (LAB), *O. oeni* has a small genome, and it is considered a rapidly evolving organism, characterized by a high level of horizontal gene transfer and high levels of recombination (36, 41, 42). In this species, some of the *pts* genes appear to contribute to strain differentiation and species evolution by being targets for indel events and new gene acquisition by horizontal gene transfer. Actually, *pts* gene sequences are highly conserved among lactic acid bacteria, and genetic exchanges necessitate DNA target recognition (43, 44).

However, most of the *pts* genes described are highly conserved among the *O. oeni* strains studied. This suggests that these genes encode proteins responsible for important steps of carbohydrate

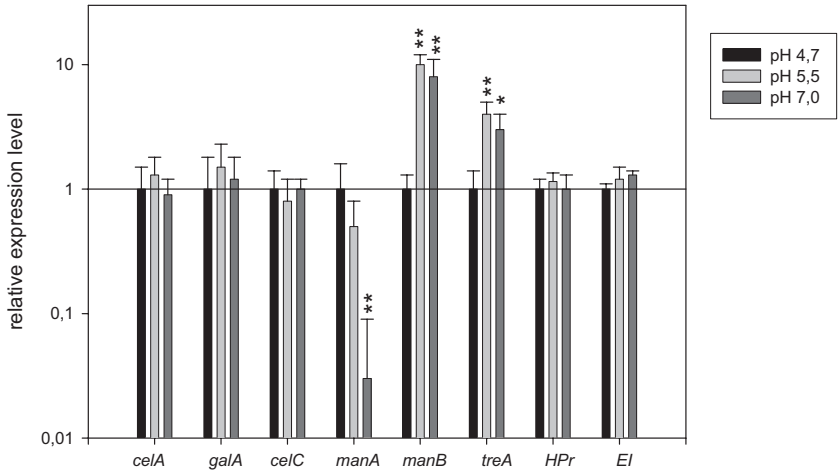

FIG 6 Influence of the pH of the medium on *pts* gene expression. *O. oeni* PSU-1 was grown in MRS-glucose medium without alcohol (initial pH 4.7, 5.5, or 7.0). The same results were obtained with MRS-cellobiose or MRS-trehalose. The PSU-1 strain grown in medium with an initial pH of 4.7 was used as the control, and the gene expression level was set at 1. The gene studied for each locus is indicated in Table 1 and Table S1 in the supplemental material. Values that were significantly different from the level of expression of the same gene in cells after growth at pH 4.7 are indicated by asterisks as follows: *, $P < 0.05$ by the Student *t* test; **, $P < 0.005$ by the Student *t* test.

TABLE 2 PTS and glucokinase activities measured with *O. oeni* PSU-1 or *L. lactis* IL1403 decryptified cells after growth on various carbohydrates and toluene permeabilization[a]

Activity	Mean PTS or glucokinase activity (nmol · min^{-1} · mg of cells^{-1}) ± SEM[b]					
	Oenococcus oeni PSU-1 grown on:					*L. lactis* IL1403 grown on glucose
	Trehalose	Cellobiose	Glucose	Mannose	Arabinose	
$PTS^{trehalose}$	4.25 ± 0.15	<0.50	1.25 ± 0.08	1.41 ± 0.15	<0.5	5.40 ± 0.50
$PTS^{glucose}$	1.25 ± 0.12	2.40 ± 0.40	2.03 ± 0.17	3.92 ± 0.25	<0.5	10.00 ± 0.50
$PTS^{cellobiose}$	<0.50	1.98 ± 0.15	1.40 ± 0.12	1.32 ± 0.12	<0.5	2.10 ± 0.15
$PTS^{mannose}$	<0.50	<0.50	1.09 ± 0.12	3.05 ± 0.25	<0.5	4.15 ± 0.25
$PTS^{2\text{-deoxyglucose}}$	<0.50	ND	<0.50	<0.50	ND	ND
Glucokinase	4.96 ± 0.50	2.75 ± 0.35	2.13 ± 0.25	2.32 ± 0.25	2.23 ± 0.26	21.00 ± 1.08

[a] All the assays where the activity was higher than 1 nmol · min^{-1} · mg cells^{-1} presented slopes significantly higher (at least 3 times) in the presence than in the absence of the indicated PTS substrate (trehalose, glucose, cellobiose, etc.).
[b] ND, not determined.

metabolism in the species. Furthermore, *pts* genes are expressed in the presence of alcohol, at acidic pH, and in the presence of grape polyphenols (grape juice assay). This suggests that they may be expressed in winemaking conditions. Moreover, when grown on the appropriate substrate, *O. oeni* is able to efficiently phosphorylate—at the expense of PEP—different carbon substrates. (i) Glucose and trehalose, which are present in a free form in wine at the end of alcoholic fermentation, are two of the PTS substrates identified. For channeling trehalose to central catabolic pathways (transport, phosphorylation, and hydrolysis), there is no evident alternative to PTS in the *O. oeni* PSU-1 genome sequence. (ii) Mannose, which can be liberated from yeast mannoproteins by *O. oeni* as a result of mannosidase activities, also appears as a PTS substrate. PTS could thus be implicated in *O. oeni* growth stimulation in the presence of yeast mannoproteins or yeast extracts (45). (iii) Cellobiose and maybe other β-glucosides present in grape juice constitute other PTS substrates for *O. oeni*. PTS activities are thus important in *O. oeni* for metabolism of glucose and of many other carbon substrates. PTS activities may be crucial for *O. oeni* settlement and development, in competition with spoilage microbial species in wine at the end of alcoholic fermentation (46, 47). PTS could also contribute to the probiotic potential of *O. oeni*, by allowing it to metabolize the substrates not absorbed in the upper part of the digestive tract, such as cellobiose (48). This opens many research perspectives, since PTS regulatory functions may contribute to improved stress tolerance or biofilm formation by *O. oeni*, as previously shown for diverse lactobacilli and streptococci (22, 23, 49).

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